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Transgenic mice expressing *Erb*B2 develop mammary tumors with a latency of over 200 days. We were interested in examining the cooperation between mutant *p53* and *Erb*B2. We examined mammary tumors arising in MMTV-*Erb*B2 transgenic mice for mutations in exons 4-8 of p53 by direct sequencing of PCR products, and have found that 37% of tumors have a missense mutation at codon 256, which converts an Asp to Asn. We have directly tested for cooperativity between *Erb*B2 and mutant *p53* in mammary tumorigenesis by creating bitransgenic mice carrying MMTV-*Erb*B2 and p53-172$^{Arg\text{-}His}$. Bitransgenic mice expressing *Erb*B2 and p53-172$^{Arg\text{-}His}$ develop mammary tumors with a latency of 154 d, where mice expressing *Erb*B2 alone develop tumors with a latency of 234 d. Tumors arising in the p53/*Erb*B2 bitransgenic mice show large cell size, marked nuclear pleiomorphism, high mitotic rate, abnormal mitotic figures, and increased apoptosis. Ploidy analysis revealed that while tumors arising in the *Erb*B2 mice were diploid, tumors arising in the p53/*Erb*B2 bitransgenic mice comprised aneuploid cells. Analysis of these tumors revealed the absence of activating mutations in the *Erb*B2 transgene, while analysis of the ErbB2 receptor shows that the receptor is highly phosphorylated. These data indicate that *p53* mutation is an important cooperating event in *Erb*B2-mediated oncogenesis.

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Archibald S. Perkins 8/29/97

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Introduction

A central goal of current cancer research is the identification of the genes involved in tumorigenesis, and the definition of the precise role that these genes play in tumor development. Analysis of human breast carcinomas has implicated a number of genes in the genesis of these tumors, including *Erb*B2[1], *HST* and *INT2* [2], *p53* [3], *src* [4], *Rb* [5]. It is suggested by a number of studies that the development of breast cancer in humans requires changes in more than one of these genes, which may in part explain the long latency associated with this disease [3].

*Erb*B2 encodes a receptor tyrosine kinase related to the receptor for epidermal growth factor (EGFR or ErbB), and is amplified in nearly 30% of human cancers, particularly intraductal carcinomas[6, 7] . Numerous studies suggest that this amplification leads to increased mitogenic signaling in the cell. The importance of this amplification is supported by the finding that 70% of transgenic mice that overexpress rat *Erb*B2 in the mammary gland develop mammary carcinomas [8]. However, the latency of tumorigenesis is relatively long (over 200 d), suggesting that other oncogenic events are necessary. Analysis of these tumors revealed small in-frame deletions in the *Erb*B2 transgene in 65% of tumors analyzed [9]. These deletions resided in the extracellular domain adjacent to the transmembrane domain, and resulted in activation of ErbB2 tyrosine kinase activity. These findings indicate that activation of ErbB2 tyrosine kinase activity plays an important role in the development of these tumors. This is consistent with previous experiments, showing that mice carrying an MMTV-driven rat *Erb*B2 transgene with an activating mutation in the transmembrane domain develop multifocal mammary carcinomas with a singnificantly shorter latency [10].

In 30% of human breast carcinomas, expression of *ERB*B2 is associated with the presence of mutant *p53*, suggesting that activated tyrosine kinase receptors cooperate with mutant p53 in the development of these tumors [3]. p53 is a multifunctional protein that is involved in the regulation of growth of nearly all cell types within mammalian organisms (reviewed in [11]. The wild type p53 protein can suppress tumor cell growth [12], and likely functions as a regulatory protein in two capacities: as a key component of apoptotis pathways within the cell [13]; and as a checkpoint protein to

control G1 to S transition in the presence of genotoxic stress [14]. Structural domains of p53 include an amino-terminal transcriptional activation domain, a central DNA binding domain, and a carboxy-terminal domain important for oligomerization (reviewed in [11]). Genetic alterations at the *p53* locus are common in human cancers, and are primarily either missense mutations or allele loss [15-17]. While the majority of human tumors with altered *p53* have one allele bearing a missense mutation and one null allele, occasionally tumors are found to have one mutated allele and one normal allele [15]. These findings suggest a progression model in which the initial event is a missense mutation in one p53 allele, leading to a proliferative advantage, and then loss of the other allele, which confers a further selective advantage.

p53 point mutations are highly clustered into four regions that correspond to evolutionarily conserved domains of the protein that function in DNA binding. Some of the most commonly mutated amino acids are those that make direct contact with the DNA [18]. p53 proteins bearing these mutations have been found to have altered DNA binding and transactivation properties [19, 20]. Some mutant proteins fail to activate normal target genes, such as p21, but can activate atypical targets, such as *MDR1* [21]. Thus, certain mutations in p53 may lead to the acquisition of novel and dominant activities within the cell. It is evident from a number of studies that certain missense mutations in p53 function as dominant negative alleles that encode proteins that lack transcriptional activation potential, but retain the ability to oligomerize and thus can pull wildtype p53 into nonfunctional complexes [22]. An example of this is the 135V mutation, which can accelerate tumor development in heterozygous but not nullizygous p53-deficient mice [23]. Other alleles, such as 143A, 175H, 248W, 248Q, 273H, and 281G act as dominant oncogenic alleles, since they can confer new malignant phenotypes upon gene transfer into cells that lack p53 [24, 25]. These phenotypes include the ability to grow in soft agar, and to form invasive tumors in nude mice. The molecular mechanisms that underlie the ability of mutant p53 alleles to induce these changes are unknown.

p53 alterations are common in human breast carcinomas [26, 27]. Missense mutations have been identified at many of the hotspot regions, including

codons 175(R to H), and 248(R to Q). 175H represents approximately 8% of all p53 mutations in human breast cancers. These alleles are dominantly oncogenic in cell culture and nude mouse tumorigenicity assays [24, 25]. To obtain a more accurate picture of the effect that the 175H allele has on mammary cell growth, we used transgenic mice in which this allele was targeted to the mammary epithelium using the whey acidic protein (WAP) promoter. It was somewhat surprising to find that, despite high level expression in the mammary gland, mice carrying the WAP-driven p53-175H were not abnormally susceptible to mammary carcinomas – only one mouse developed a mammary carcinoma and this was with a latency of 11 months [28]. These data suggested that this allele is not dominantly oncogenic on its own in this setting, and requires other cooperating events. Indeed, these mice were much more susceptible than nontransgenic control mice to mammary tumors induced by carcinogens that are known to activate Ha-Ras [28-30]. This suggests that activated Ras is one molecule that can cooperate with p53-172H in this system.

It is known that ErbB2 can initiate a mitogenic signal within the cell, and that this signal utilizes the same pathway as activated ras. This suggested that if p53-172H can cooperate with activated Ras, it may also cooperate with ErbB2. In this study, we demonstrate cooperativity between ErbB2 and p53-172H in the development of mammary carcinomas, and offer this as a model system that closely mimics the genetic changes in human breast cancers, and that allows for further studies to uncover the mechanism of cooperativity between these two genes.

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Experimental Methods

Transgenic Mice The *p53-172H* transgenic mice, in which mutant *p53* transgene was preferentially overexpressed in the mammary epithelium by use of the whey acidic protein (WAP) promoter, were created and characterized as described [28]. Unactivated *Erb*B2 transgenic mice (line N#202) which contain the wild type rat *Erb*B2 gene driven by MMTV have been described previously [8]. Both lines are on an FVB background. *p53/Erb*B2 bitransgenic mice were generated by crossing female and male offspring of line 8512 *WAP-p53-172H* transgenic mice to offspring of line N#202 of *MMTV-Erb*B2 transgenic mice. Mouse tail DNA from the offspring of this cross was isolated as described previously [31]. Mice carrying both WAP-p53 and *MMTV-Erb*B2 transgenes were identified by multiplex PCR. The screening primers for *p53* transgene utilized a 5' primer on the *WAP* promoter (5'-CCGTCGACGGCCACAGTGAAGACCTCCGGCCAG-3'), and a 3' primer on exon 2 of murine *p53* (5'-GCCTGAAAATGTCTCCTGGCTCAGAGGG-3') and yielded a 1.2 kb PCR product. Primers for the rat *Erb*B2 cDNA [(5'-GGAAGTACCCGGATGAGGAGGGCATATG-3') and (5'-CCGGGCAGCCAGGTCCCTGTGTACAAGCCG-3')] were used to identified *Erb*B2 transgenes, yielding a 0.7 kb PCR product, which corresponds to nucleotides 1872 to 2578 of rat *Erb*B2 cDNA. PCR primers for mouse *β-casein* exon 7 [(5'-GATGTGCTCCAGGCTAAAGTT-3') and (5'-AGAAACGGAATGTTGTGGAGT-3')] provided an internal control for the PCR reaction. The PCR reaction (100 µl volume, containing 2.5 mM $MgCl_2$, 1 x PCR buffer (Promega), 0.2 mM of each dNTP, 0.1 µM of each primer, 2.5 U. Taq polymerase (Promega) and 2.0 µg template DNA) consisted of 31 cycles of 1 min and 15 sec at 94°C, 2 min and 15 sec at 60°C, and 3 min and 15 sec at 72°C (RoboCycler Gradient 40, Stratagene). PCR positive *p53/Erb*B2 bitransgenic mice were confirmed by Southern blot analysis as described previously [32].

Screening of p53 mutation in ErbB2-induced mammary tumors DNA extracted from MMTV-ErbB2-induced mammary tumors were subjected to PCR reaction to amplify exon 5-6 and exon 7-8 of murine p53 gene for sequencing. The primers for amplying exon 5 and 6 were 5'CGTTACTCGGCTTGTCCCCGACCT-3' and 5'-CAACTGTCTCTAAGACGCACAAC-3' (which reside on introns 4 and 6 of murine *p53* respectively). The primers for

amplying exon 7-8 were 5'-GAGGTAGGGAGCGACTTCACCTGG-3' and 5'-TGAAGCTCAACAGGCTCCTCCGCCTCC-3' (on intron 6 and 8 of murine p53 gene respectively).

RNA Extraction and Analysis Mammary gland and mammary tumor biopsies were performed under anesthesia (Avertin, i.p.) as described previously [33]. Tissues were frozen down immediately in liquid nitrogen and kept at -80°C until performance of RNA isolation. RNA was isolated by homogenization of frozen tissues with a homogenizer (Janke & Kunkel KIKA-Labortechnik) using the TRIzol protocol as described by the manufacturer (GIBCO BRL). RNA was fractionated by electrophoresis in a 1.2% agarose gel containing 0.66 M formaldehyde with 1 x 4-morpholinepropanesulfonic acid buffer, then transfered to Zetaprobe membranes (Bio-Rad) with 10 x SSC and hybridized as described [32], using an *Xho*I-*Kpn*I fragment excised from mouse *p53* cDNA as probe to detect the expression of *p53* transgene, and a BamH I-BamH I fragment excised from rat *Erb*B2 cDNA as probe to detect the expression of *Erb*B2 transgene.

PCR analysis of deletion on *Erb*B2 transgene in mammary tumors DNA was isolated from mammary tumors of *Erb*B2 transgenic mice and *p53/Erb*B2 bitransgenic mice as described previously [34]. Hot-start PCR was used to analyze deletions in *Erb*B2 transgenes in mammary tumors. The PCR was performed with the following primers: 5'-CGGAACCCACATCAGGCCCTGCTCCACAGT-3' and 5'-CTCAGTTTCCTGCAGCAGCCTA CGCATCG-3', which amplify the region corresponding to nucleotides 1487 to 2116 of rat *Erb*B2 cDNA, and yield a 629 bp PCR product. The forward primer was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The PCR conditions used were the same as those screening bitransgenic mice, but used 1.5 µg of tumor DNA as template. Six µl of labeled PCR products were mixed with 4 µl of Sequenase stop buffer, and then heated to 75°C for 5 min. Five µl of this mixture was separated by electrophoresis through 5% polyacrylamide sequence gels and exposed to X-ray film.

Immunoprecipitation and immunoblotting Tissue lysates were prepared as described previously [9]. Immunoprecipitation were performed by incubating 500 µg of the cleared protein lysate with 500 ng of anti-ErbB2 antibody

(Ab-4, Oncogene Science) for 2 h at 4°C, with 4,600 ng of rabbit anti-mouse IgG for 1 h at 4°C and then with protein A sepharose for 1 h at 4°C on a rotating platform. ErbB2 immunoprecipitates were washed three times with lysis buffer and resuspended in 75 µl SDS gel loading buffer. Fifty µl of each sample was electrophoresed on 7.5% SDS gel. After being electrophorested, the protein was transferred onto a Biotrace NT membrane (Gelman Sciences) with an immunoblot transfer (Hoefer). The membrane was blotted with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) first, stripped and re-blotted with anti-ErbB2 antibody (sc-284, Santa Cruz Biotechnology). Proteins were visualized with ECL kit (Amersham).

Histologic analysis Mammary glands and mammary tumors were surgically removed, fixed in 10% neutral buffered formalin (ANATECH LTD, Battle Creek, MI) for 6 h, and placed in 70% ethanol until processed. These tissues were embedded in paraffin, and 5 µm sections were placed on regular slides and stained with hematoxlin and eosin.

Flow cytometry DNA content of mammary tissues was analyzed by flow cytometry on paraffin sections as described previously[35]..

Results and Discussion

Analysis of the *p53* gene in MMTV-*Erb*B2-induced tumors reveals mutations in p53. Mice carrying *MMTV-Erb*B2 (line N#202) express *Erb*B2 at high level in the mammary gland and develop mammary tumors with a latency of seven to eight months [8]. We wished to determine if mutations in p53 could be a cooperating event in the genesis of these mammary tumors, and may help to explain the long latency of tumor development. To that end, we examined eight mammary tumors arising in these mice for the presence of mutations in exons 5-8 of *p53*. These tumors have no alterations in *ErbB2* transgene. We performed direct sequence analysis of two different amplification products obtained by PCR using primers bracketing exons 5-6 or exons 7-8. Three out of 8 tumors showed a G to A transition at codon 256 in exon 7, which changed the coding potential from Asp to Asn. The remaining five tumors showed no changes in DNA sequence within the interval examined.

Expression of 172H mutant *p53* and unactivated *Erb*B2 in the mammary gland of transgenic mice. The finding of *p53* mutations in mammary tumors arising in *MMTV-Erb*B2 transgenic mice argues that p53 mutation can be a cooperating event in ErbB2-induced tumors in this model, and is thus consistent with data from the analysis of human tumors [3]. To further test this cooperativity, we sought to coexpress both genes in the mammary epithelium of transgenic mice and to determine the effect of this coexpression on susceptibility to mammary carcinomas. We previously developed a line of transgenic mice (line 8512) in which murine 172H mutant *p53* was targeted to express in the mammary gland under control of the rat whey acidic promoter (*WAP*) promoter [28]. WAP is a prominent constituent of rodent milk; its expression is restricted to the mammary gland, where it is normally turned on at day 10 of pregnancy, remaining elevated through lactation [36, 37]. Codon 172 of murine *p53* gene is equivalent to codon 175 of human *p53* gene [38, 39] and the majority of *p53* mutation on codon 175 in primary mammary tumors were found to be Arg to His [16, 26, 40]. Overexpression of murine p53-172H in the mammary gland of transgenic mice induced a mammary tumor in only one out of five female founders, with a latency of eleven months, and no other tumors have been observed in F1-F3 generation mice despite multiple breeding over more than two years. However, when the mice were treated with DMBA, mammary tumors developed with shorter latency compared to nontransgenic mice [28]. The fact that overexpression of *p53*-172H alone rarely causes mammary tumors but can markedly accelerate mammary tumor formation with DMBA treatment suggestes that an initiating event, or elevated signaling from a mitogenic pathway, was needed to cooperate with *p53*-172H for mammary tumorigenesis. We postulated that the MMTV-*Erb*B2 transgene could provide such a stimulus.

To directly test for cooperativity between *Erb*B2 and *p53*-172H in mammary tumorigenesis, we generated *p53/Erb*B2 bitransgenic mice in which both transgenes were expressed in the mammary gland. *p53* transgenic mice were mated to line N#202 *MMTV-Erb*B2 transgenic mice, and *p53/Erb*B2 bitransgenic offspring were identified by DNA analysis. A total of twenty-six female *p53/Erb*B2 bitransgenic mice, twenty-five *p53*-172H alone and twenty *Erb*B2 alone female transgenic mice were identified from same group of offspring. All transgenic mice were kept either pregnant or lactating

by continued housing with male FVB mice, in order to maintain expression of *WAP*-driven transgene. To confirm coexpression of *p53*-172H and *Erb*B2 transgenes, we performed Northern blot analysis of RNA from mammary gland biopsies performed at 2 days postpartum during lactation from five *p53/Erb*B2 bitransgenic mice. This analysis showed that both *p53* and *Erb*B2 mRNA were readily detected in 20 μg of total RNA after an 16-h exposure, with some variability in different individuals.

Development of mammary tumors is accelerated in *p53*-172H/*Erb*B2 bitransgenic mice. At 112 days of age, after two rounds of pregnancy and lactation, mammary tumors began to appear in the *p53*-172H/*Erb*B2 bitransgenic mice. In the *Erb*B2 alone transgenic mice, mammary tumors began to emerge at 163 days of age. At age of more than 300 days after three and four rounds of pregnancy, no tumors had appeared in *p53* alone transgenic mice. The median age of tumor development was 154 d for *p53/Erb*B2 bitransgenic mice, whereas it was 234 d for *MMTV-Erb*B2 singly transgenic mice. These data indicate a strong cooperation between *Erb*B2 and the dominant oncogenic 172H allele of *p53*.

To check the expression status of *p53* and *Erb*B2 transgenes, RNA isolated from both mammary tumor and adjacent mammary gland of *p53/Erb*B2 bitransgenic mice were subjected to Northern analysis. The expression levels of p53 appeared reasonably constant in both mammary tumors and adjacent mammary glands from three different *p53/Erb*B2 bitransgenic mice, and were similar to the level of nonneoplastic mammary gland from singly transgenic p53-172H mice. However, the expression of *Erb*B2 transgene in mammary tumors was much higher than in adjacent mammary gland, but the level of expression did not appear to correlate with the presence or absence of the *p53*-172H transgene.

Tumors with p53-172H exhibit a higher grade and have higher rates of mitosis and apoptosis. Histological examination of the tumors revealed that the presence of the *p53*-172H transgene had a marked effect on tumor morphology. Tumors arising in the *MMTV-Erb*B2 singly transgenic mice are typical mammary adenocarcinomas, exhibiting focal gland formation, solid clusters of tumor cells, and abundant tumor angiogenesis. While the

nuclear-to-cytoplasmic ratio was high, nuclear size was rather uniform, and the majority of tumor cells had smooth nuclear borders. In contrast, the *p53*-172H-expressing tumors arising in the bitransgenic mice had a much larger cellular and nuclear size, pronounced anaplasia, and, as assessed by morphology alone, a markedly higher rate of both apoptosis and mitosis. Nuclear shape was markedly irregular, exhibiting nuclear grooves, folds, and lobulations. These features are consistent with a much higher grade of neoplasm, with a higher growth fraction, and suggest aneuploidy or polyploidy. Thus, the expression of *p53*-172H in this setting appeared to have a marked effect on tumor cell morphology and tumor growth.

From the histologic appearance it is evident that tumors expressing *p53*-172H have a higher rate of apoptosis and mitosis. To confirm this, we assayed the relative rate of mitosis with BrdU labeling, and the apoptosis index with the TUNEL assay. We injected *Erb*B2 singly transgenic and *p53/Erb*B2 bitransgenic mice harboring equal sized-tumors in parallel with BrdU, sacrificed the mice, and immunostained the mammary tumors for BrdU incorporation into DNA. The results show a clearly higher mitotic rate in tumors expressing *p53*-172H than those without. We also assessed the rate of apoptosis on similar tumor samples, as well as nonneoplastic mammary glands from two mice of the genotypes under study, and these results show a markedly higher rate of apoptosis in mammary tumors expressing both p53-172H and *Erb*B2. These data confirm the impression obtained from examination of the H+E-stained slides. The bitransgenic tumor also showed a higher apoptosis rate than the adjacent nonmalignant tissue. Interestingly, premalignant mammary glands from bitrangenic mice had a significantly higher rate of apoptosis than similar tissue from *Erb*B2 transgenic mice.

Bitransgenic tumors exhibit aneuploidy and tetraploidy. On the basis of the large nuclear size seen on the H+E-stained sections of the most mammary tumors arising in bitransgenic mice, we suspected that the tumors expressing p53 had greater than 2n DNA content. We thus investigated the ploidy of the premalignant and malignant mammary tissue by flow cytometry of nuclei derived from paraffin-embedded tissue. We first determined the ploidy of cells in nonmalignant mammary cells, on the 2nd

day of lactation, and found all genotypes to have 2n DNA content, with a similar fraction of cells in G2/M and S. In tumor specimens, while *Erb*B2 alone tumors were euploid, all four bitransgenic tumors analyzed were markedly aneuploid, with a minority of cells having 2n DNA content: the majority had 4n DNA, or were intermediate in DNA content. In collaboration with Allan Coleman and Thomas Reid of the NIH, we are in the process of doing chromosome painting, also known as spectral karyotying [41, 42] on tumors arising in the singly and doubly transgenic mice, and have found that in one p53-172H/ErbB2 tumor, there is an amplification of mouse chromosome 5, band E1, which is the location of the *Fgf5* locus. We are currently pursuing the possible involvement of *Fgf5* in mammary tumor progression in these mice.

Bitransgenic tumors exhibit increased ErbB2 tyrosine phosphorylation. We were interested in exploring the mechanism of p53-induced tumor acceleration in the bitransgenic mice. One possible role of p53 is to alter the intrinsic tyrosine kinase activity of ErbB2, through either a direct or indirect effect. Muller and coworkers have found that the induction of mammary tumors in transgenic mice expressing the unactivated *Erb*B2 alone is associated with activation of the receptor's intrinsic tyrosine kinase activity [8]. To determine if this was the case with mammary tumors arising in the bitransgenic animals, we performed immunoprecipitations using anti-ErbB2 antisera followed by Western blot analysis using antisera against either phosphotyrosine or ErbB2. This analysis revealed an elevation in levels of ErbB2 protein in all of the bitransgenic tumors relative to that in the adjacent nonmalignant mammary gland, and in five tumors, the level is comparable to that seen in *Erb*B2 singly transgenic mice. In addition, the level of tyrosine phosphorylated ErbB2 in these tumors is comparable to *Erb*B2 alone transgenic tumors. However, the level of ErbB2 expression in the bitransgenic tumors is not as consistent as that seen in this sampling of *Erb*B2 singly transgenic tumors. There also appears to be little correlation between the level of ErbB2 protein in the tumor and the level of tyrosine phosphorylation, for either set of tumors. Nonetheless, these data indicate that, as in the singly transgenic tumors, the ones arising in *p53* positive bitransgenic mice exhibit elevated levels of tyrosine-phosphorylated ErbB2.

Deletions of *ErbB2* transgene are not detectable in mammary tumor of *p53/Erb*B2 bitransgenic mice One mechanism of ErbB2 activation in mammary tumors arising in MMTV-*Erb*B2 mice is through small (7-12 aa) somatic deletions in unactivated *Erb*B2 transgenes [9]. The finding of these mutations in 65% of the tumors argues that activation of ErbB2 tyrosine kinase activity is a rate-limiting step in tumor development. We wondered if the presence of the *p53*-172H transgene abrogated the need for these activating mutations in the *Erb*B2 transgene, and thus we analyzed tumor RNA and DNA for the presence of activating deletions of the *Erb*B2 transgene. The RNA was subjected to RT-PCR analysis using radioactive primers that generated a fragment spanning from nucleotide 1487 to 2116 of rat *Erb*B2 cDNA which is the region where deletions of *Erb*B2 transgene were found in MMTV-*Erb*B2-induced mammary tumors [9]. DNA samples were subjected to PCR with same primers. Both RT-PCR and PCR results revealed that the deletions of *Erb*B2 transgene did not occur in the mammary tumors of *p53/Erb*B2 bitransgenic mice, while a deletion was detected in the DNA and RNA from a mammary tumor that arose in a MMTV-*Erb*B2 singly transgenic mouse. This suggest that unlike *Erb*B2 alone-induced mammary tumors, *Erb*B2 deletions are not associated with the mammary tumor formation in *p53/Erb*B2 bitransgenic mice, and suggests that the presence of the *p53*-172H allele abrogates the need for these mutations.

Higher levels of TGFα were detected in the mammary tumors of *p53/Erb*B3 bitransgenic mice Unlike mammary tumors from *ErbB2* alone transgenic mice, no somatic deletions were detected in *ErbB2* transgene from the mammary tumors of *p53/ErbB2* bitransgenic mice. One possible mechanism is activation via ligand stimulation. We have addressed this possibility by assessing the level of expression of several ligands known to activate ErbB2 through transmodulation. Northern blot analysis of TGFα expression in the mammary glands and the mammary tumors from p53, *ErbB2* and p53/*ErbB2* mice revealed a higher levels of TGFα expression in the mammary tumors of p53/*ErbB2* bitransgenic mice relative to nonmalignant mammary tissue of the same genotype, or to tumor tissues of the other genotypes.

Conclusions

This report describes the creation of a mouse mammary tumor model in which two of the most frequent changes in human breast cancers – amplification of *Erb*B2 and a dominant oncogenic mutation of *p53* – have been recapitulated. This model serves to address two important issues in tumor development: the mechanism of cooperation of genes in mammary tumorigenesis, and the effect of dominant oncogenic alleles of *p53* on tumor growth in an in vivo experimental model.

To address the possibility that *p53* mutations play a cooperating role in *Erb*B2-mediated mammary tumors, we document the presence of *p53* point mutations in 3 out of 8 mammary tumors that arose in MMTV-*Erb*B2 transgenic mice. To directly address a genetic interaction between *p53* and *Erb*B2, we then crossed MMTV-*Erb*B2 transgenic mice with mice transgenic for the dominant oncogenic *p53*-172H allele (equivalent to the human 175H allele). Strikingly, while we observed only a single mammary tumor out of twentyfive 172H transgenics, we found strong cooperation between the 172H allele and MMTV-*Erb*B2. We further show that unlike tumors induced by MMTV-*Erb*B2 alone, the 172H+*Erb*B2 tumors exhibit no activating deletions in the *Erb*B2 transgene. Nonetheless, the tumors have increased tyrosine phosphorylation of the ErbB2 protein, indicating receptor activation. This indicates that the presence of the dominant oncogenic *p53* allele abrogates the need for activating mutations of *Erb*B2 in mammary tumorigenesis. It is unlikely that the etiology of the increased ErbB2 receptor activity is a direct effect of 172H, since the nonmalignant bitransgenic mammary tissue does not exhibit it. Thus, this feature emerges during tumorigenesis.

It is known that dominant oncogenic mutants of p53 such as 175H can cause immortalization of primary cells [43], can cooperate with Ras in transforming primary cells [44, 45], and can enhance the tumorigenic potential of cells lacking p53 [24]. 175H is particularly potent, being able to induce growth of SAOS-2 cells in agar, where other mutant alleles are not [24]. The rapid kinetics and high efficiency of cooperation in these assays by dominant oncogenic alleles of p53 indicate a direct effect on tumor cell growth. That these effects can be seen in the absence of endogenous p53 argues that these alleles are not acting simply as dominant negative alleles, by inactivating wildtype p53 function. These features of cellular

transformation mediated by mutant p53 alleles suggest that these alleles act not only by interfering with p53-dependent functions such as apoptosis, senescence, or genomic instability, [all of which have been suggested as important tumor-promoting sequelae of p53 loss [46-49]], but also by exerting a dominant effect on cell growth. The nature of this effect is unknown. Recent data from skin tumorigenesis studies in mice support the distinction between p53 null alleles and dominant oncogenic mutations. TPA-treated transgenic mice specifically expressing TGFα in the skin develop skin tumor with about 8 weeks latency. The onset of skin tumor was delayed in p53-/- mice bearing the same TGFα transgene [50]. However, in p53-172H/TGFα bitransgenic mice, the latency of TPA-induced skin tumors was shortened to 3-4 weeks (Wang, X-J et al, personnel communication).

In our bitransgenic model, we do not observe the emergence of tumors with kinetics that indicate direct and immediate malignant transformation by coexpression of 172H and *Erb*B2: tumors arise following the second pregnancy rather than the first, and are unifocal, indicating the necessity for other events. This is thus distinct from the cell culture results described above, and is likely due to several things, including the lower transforming potential of native *Erb*B2 relative to Ras, the presence of endogenous p53 alleles in our transgenic mice, as well as other tumor control mechanisms that exist in the intact animal, such as tumor immunity, the inhibitory influence of surrounding tissue, and the requirement for tumor angiogenesis. Nonetheless, the 172H allele accelerates *Erb*B2-induced tumorigenesis, albeit by an unknown mechanism. We present several possible mechanisms that our bitransgenic model will allow us to address. These models are based on the known or suggested functions of p53, which include an effect on apoptosis, on genome stability, and on transcriptional regulation of cell growth regulatory genes.

A role for p53 in programmed cell death is well established, and is likely mediated through its ability to transcriptionally activate the cell death agonist, *bax* [51]. It has been proposed that the loss of p53-mediated cell death is an important tumor-promoting mechanism in p53-/- tumors [48]. Tumors that arise in one SV40 T antigen model exhibit lower levels of apoptosis relative to control tumors, suggesting that p53 plays an

essential role in apoptosis [48]. The 135V allele, which acts as a dominant negative allele, can block E1A-induced apoptosis [52]. However, this allele cannot cooperate with ErbB2 in mammary carcinogenesis (Muller, W.J. et al., unpublished results), which suggests that one cannot accelerate *Erb*B2-induced murine mammary tumorigenesis by decreasing apoptosis. Similarly, p53-dependent apoptosis in the mammary cells appears not to be required for normal mammary gland development [53, 54]. Our data indicate an increased rate of apoptosis 172H-induced tumors, making the loss of apoptotic cell death an unlikely mechanism for 172H cooperativity in mammary tumorigenesis.

In *Erb*B2-alone tumors, activation of ErbB2 through mutations in the *Erb*B2 transgene is an important, rate-limiting step in tumorigenesis [9]. It is likely that ErbB2 activation is also rate limiting in the p53-172H x *Erb*B2 bitransgenic tumors. Thus, understanding the mechanism underlying this increase in RTK activity is a possible key to understanding the role of 172H in accelerating tumor formation in this model. By Northern blot analysis, we have documented that both the *Erb*B2 transgene, and the activating ligand *TGFα* are expressed at higher levels in the bitransgenic tumors than in nonmalignant mammary tissue of the same genotype. These data provide two possible mechanisms for increased ErbB2 RTK activity, but are not likely to be direct effects of 172H, given the low expression of the genes in nonmalignant bitrangenic tissue. Thus, these changes in the level of *Erb*B2 and *TGFα* gene expression may be accompany malignant progression rather than cause it. We are currently determining if other ligands for the ErbB family of receptors may be transcriptionally altered in a direct manner by 172H. An alternative mechanism is that 172H could cause an increase in the expression of a receptor critical for ErbB2 function, such as EGFR, ErbB3, of ErbB4.

The data from cell culture experiments described above suggest a direct effect of 172H on tumor cell growth, and such an effect may indeed play an important role in our system. However, other effects of this allele are also possible. One is that p53-172H increases the likelihood of additional mutational events in genes other than the *Erb*B2 transgene in the nonmalignant cells expressing MMTV-*Erb*B2, and thus accelerates tumor formation. One type of genetic alteration known to contribute to mammary

tumorigenesis is gene amplification. While an increased frequency of gene amplification is seen in p53 null cells, it is not observed in Li Fraumeni cells (mutated at 184 or 248) that retain one wild-type p53 gene [55] Since our 172H+*Erb*B2 bitransgenic tumors appear by Southern blot analysis to retain (a) wildtype copy or copies of p53 (data not shown), this mechanism may not apply to this model. We are currently assessing the frequency of other types of alterations – e.g., deletions, point mutations – in these bitransgenic tumors.

One notable feature of the tumors expressing p53-172H is their large nuclear size and >2n DNA content, which occurred despite the retention of the endogenous wildtype p53 allele(s). Aneuploidy was found by some investigators in tumors driven by p53 null alleles [56], and in primary p53-/- fibroblasts following extended culture [55, 57, 58] but not in primary p53-/- hematopoietic cells, or in p53-/- erythroid tumors, or in the majority of cell lines derived from these tumors, even following 150 passages [49]. It is known that polyploid nuclei can result from the uncoupling of S phase and mitosis. One way in which this can occur is through loss of the p53 target gene, *p21*, which encodes a negative regulator of cyclin-dependent kinases. In the absence of p21, or in the presence of mutant p53 (in which case p21 is not induced by DNA damaging agents), cells fail to arrest at G1/S and will replicate their DNA. Cells then proceed into additional rounds of DNA replication and culminating in apoptosis [59]. At a low frequency, this can occur in p53-/- cells in the absence of DNA damaging agents [49]. This p53- and p21-dependent G1/S checkpoint may play an important role in vivo to arrest cell growth in the setting of tumor hypoxia [60], and the loss of this pathway may then result in chromosomal reduplication, a hallmark of malignant tumors [59], and a feature of tumors expressing p53-175H. p53 is also thought to play an important role in centrosome duplication. In p53-/- mouse embryo fibroblasts, multiple copies of functionally competent centrosome are generated during a single cell cycle, which is thought to result in unequal segregation of chromosomes [61]. These data suggest that loss of wildtype p53 function may cause chromosomal instability. It is important to note that in our system, aneuploidy does not arise prior to tumor formation, indicating that either 1) other genes need to be mutated

in order to allow polyploidization; or that 2) epigenetic events, such as tumor hypoxia, must occur [62].

An alternative mechanism of *p53*-172H action in this model is that it may promote other aspects of tumor growth, such as tumor angiogenesis. The finding that mutant, but not wild-type, p53 can synergize with PKC to stimulate vascular endothelial growth factor (VEGF) [63], suggests that the 172H allele could stimulate vascular ingrowth, which is known to be a rate-limiting step in tumorigenesis. Another potential mechanism to explain the cooperativity between *p53*-172H and *Erb*B2 is that the mutant p53 may have a negative effect on the antiproliferative signaling of TGFβ, a factor that can cause slowing of growth, G1 arrest, or apoptosis, depending on the cell line. TGFβ inhibition of cell growth can be observed in p53 null cells [64], and in cells expressing the E6 gene of human papilloma virus, which causes the degradation of p53 protein [65], indicating that wild-type p53 does not play a role in TGFβ signaling. However, lack of responsiveness to TGFβ has been correlated with certain mutations at p53 [66], and transfer of mutant p53 alleles, either murine 132F[67], 135V[68, 69], or a human 143A [70] causes reduced responsiveness to TGFβ in some cells but not others [71, 72]. These data suggest that dominant oncogenic alleles of p53 may act to interfere with TGFβ signaling, either through a decrease in TGFβ type I or type II receptor, or through interference with intracellular TGFβ signaling. Specifically, TGFβ has been shown to decrease cdk4 levels, and mutant p53 can block this effect [69].

Summary We have created a mouse model for human breast cancer closely mimics the genetic changes that occur in the human disease. Twenty-five to 30% of human breast cancers show amplfication and overexpression of *Erb*B2 gene, and of these, many will have point mutations in p53 [3]. The 175H mutation is the most common p53 mutation in human breast cancers, and is often accompanied by loss of the other allele, arguing that it is not simply acting as a dominant negative [73]. Thus, we have created a useful model for the study of human breast cancer.

Relationship to Statement of Work

Task 1 We are well underway towards the completion of this task. We have completed the analysis of the genetic interaction of ErbB2 and p53-172H. We are currently setting up crosses to examine the interaction between ErbB2 and the p53 null allele, as well as to determine if the p53-172H allele functions as a dominant oncogenic or as a dominant negative allele. We have decided not to pursue the crosses with mice overexpressing TGF α, as this is being done by another group. The crosses with the NDF-overexpressing mice still have to be done.

The proviral tagging screen for cooperating oncogenes is well underway. We have crossed the p53-172H allele onto the C3H background, and are generating mice that have both MMTV and p53-172H. To date, 5 such mice have been generated.

Task 2 Tumor incidence has been assessed on the p53-172H/ErbB2 mice, and will be determined for the other crosses as they are generated.

Task 3 Histopathology and southern analysis of the p53-172H/ErbB2 mice has been completed, as has transgene expression analysis. This will be done on the crosses that are underway.

Task 4 Biochemical analysis of tumors that have arised to date has been acomplished, and will proceed as new tumors come up.

Task 5. The cloning of novel sites of insertion has not been done, as it awaits the development of tumors in the MMTV + p53-172H mice.

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